Annals of Clinical and Analytical Medicine
Original Research

Determination of whole-genome expression differences in larynx cancers

Expression differences in laryngeal cancer

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This study was presented as an oral presentation at the 12th National Medical Genetics Congress of Turkish Society of Medical Genetics, İzmir, Turkey, 2016

Abstract

Aim: More than 200.000 new laryngeal cancer cases have been diagnosed worldwide, annually. The incidence and prevalence have increased during the past decades. It also has a high mortality rate. To have a better knowledge about this particular cancer type, we aimed to conduct a comparative analysis of whole genome expression differences between laryngeal squamous cell carcinoma and adjacent normal tissues

Material and Methods: Tissue samples were collected from specimens of laryngeal carcinoma and normal epithelium tissues adjacent to the carcinoma. RNAs isolated from these specimens were used for genome-wide gene expression analysis using microarrays. Genes that were expressed significantly differently in the tumor sample compared to normal tissues were identified. Pathway analysis of these genes was also performed.

Results: It was observed that 24 genes were significantly differentially expressed in cancer tissue. Expressions of MMP1, MMP12, S100A2, S100A3, CK14, CK16, SLC2A1, ITGA6, CEP55, KLK6, LAMC2, IL1F9, TP63, VSNL1, CXCL1, COL4A5, COL4A6, FSCN1, KRT6B, KRT17, WDR66 and ACOT7 genes were increased, while expressions of ITM2A and CFD genes were decreased.

Discussion: Some of the genes mentioned above are known to be involved in the etiology of laryngeal cancer, but to the best of our knowledge, 10 genes have been associated with laryngeal cancer for the first time in our study. These genes might be useful as biomarkers in the clinic, for early diagnosis, prognosis and personalized targeted therapy.

Keywords

Genes Expression, Laryngeal Cancers, Microarray

DOI: 10.4328/ACAM.21602 Received: 2023-01-20 Accepted: 2023-02-25 Published Online: 2023-03-11 Printed: 2023-03-25 Ann Clin Anal Med 2023;14(Suppl 1):S31-36
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This study was approved by the Ethics Committee of Necmettin Erbakan University (Date: 2015-03-13, No: 2015/146)

Introduction

Laryngeal carcinoma remains a considerable cause of morbidity and mortality with ~180,000 new cases and 99,000 deaths, accounting for 50% of mortality worldwide in 2020 [1]. Several genetic and environmental factors have been identified to be associated with the development and progression of laryngeal squamous cell carcinoma (LSCC). Among them, the major etiological agents are tobacco and alcohol consumption. In recent years, these agents have been shown to cause molecular changes and a sequence of events in cells, including oncogene activation, the inhibition of tumor suppressor genes, and changes in the gene expression profiling. Clinical outcomes in laryngeal cancer patients are thought to be based on clinicopathological features; however, few studies have indicated that gene expression alterations, as well as genomic and epigenetic changes, can also be used to predict prognosis. Due to this reason, the molecular characterization of laryngeal cancers seems to be extremely important. Certain success has been achieved in molecular studies on the etiology of larynx cancer. But previous studies on larynx cancers were uncertain and incomprehensive [2,3].

Lately, gene expression profiling (GEP) using microarrays has been identified as a hopeful method to identify variations occurring in the pathophysiology of complex diseases such as cancers. The identification of gene expression changes associated with laryngeal cancer gives us information about the mechanism of the disease and the pathways associated with this disease. Thus, new biological and prognostic markers can be identified [4].

There are many studies in the literature aimed at clarifying the etiology of the disease. But the pathogenesis of this neoplasm and associated pathways are still not fully understood. The lack of significant improvement in survival rates over the past 25 years is proof of this [5]. That is why we aimed to conduct a comparative analysis of whole genome expression differences between laryngeal squamous cell carcinoma and adjacent normal tissues. These efforts will ensure a better understanding of molecular mechanisms underlying laryngeal carcinoma, as well as enable the identification of novel biomarkers and therapeutic targets.

Material and Methods

Tissue Samples

Fresh tumor and adjacent normal tissue biopsy specimens were taken from twelve patients who were diagnosed with larynx cancer between March 2015 and April 2016. Clinical characteristics of the patients are shown in Table 1. These tissues were placed in RNA Later solution and stocked at -80 C until use. The informed consent form was obtained from patients or their families. This study was approved by the ethics committee of Necmettin Erbakan University (decision number 2015/146) and financial support was provided by the Scientific Research Projects of the Necmettin Erbakan University (Project Number:151518008).

RNA isolation

RNA isolation was performed by Norgen Total RNA Purf. Kit (cat. no. 25700). The RNA concentration was detected by Thermo Scientific Nonodrop 2000c. Then, RNA quality and quantity were identified by Agilent RNA 6000 Nano Kit (reorder-no 5067-1511). Thus, RNA was purified from other molecules with the same absorbance as itself.

Synthesis of cDNA and In Vitro Transcription of Biotin-cRNA

The Obtained RNA was converted to cDNA using the TargetAmp™-Nano Labeling Kit (Illumina® Expression BeadChip®). .before the hybridization step.''TargetAmp™-Nano Labeling Kit (Illumina® Expression BeadChip®). First-strand cDNA synthesis was catalyzed by Super Script III Reverse Transcriptase. The produced cDNA was converted to a double-stranded cDNA for use in the subsequent in-vitro transcription reaction. Then, in vitro transcription was performed for obtaining biotinylated cRNA. The synthesized biotin-labeled RNA was cleaned up again (Norgen RNA Clean-Up and Conc. Micro Kit 23600,43200) and cRNA levels of all samples were equalized before being sent to the microarray platform.

Microarray analysis

The biotin-labeled RNA was passed through some stages, including hybridization, washing, labeling with streptavidin, and drying before installing the device. The labeled RNA strand was hybridized to the bead on the BeadChip (HumanHT-12 v4.0 Exp. BeadChip Kit) containing the complementary gene-specific sequence. This kit presents a comprehensive analysis of genome-wide expression. Each channel on the BeadChip contains approximately 47,000 different bead types distributed throughout the entire genome. Also, the entire contents of the kit were created with reference to the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq) database. Hybridization was performed at 58 C for 20h. BeadChips were washed after the overnight hybridization. Thus, RNAs not bound to the probe were removed. Then, BeadChip was labeled with streptavidin. The BeadChips were immediately centrifuged after removal from the wash to prevent surface evaporation. Once the BeadChips were dry, they were stored in a dark and ozone-free environment until they were ready to be scanned. Finally, the BeadChips were read in a microarray laser reader (The Illumina® Whole-Genome Gene Expression Direct Hybridization Assay system).

Bioinformatics analysis

Preliminary analyzes and quality controls of the obtained data were performed using the 'Genome Studio' software of the Illumina iScan Microarray system. Fold change (FC) is a strong marker indicating the statistical significance of the genes, which is observed in the gene expression profile. By FC analysis, larger than two- fold changes were accepted as an increase and decrease for each probe. Heat-map analysis for differentially expressed genes was generated and GO (gene ontology) analysis was performed.

Ethical Approval

Ethics Committee approval for the study was obtained.

Results

In this study, we identified that 14,294 genes were differentially expressed between laryngeal squamous cancer tissues and non-neoplastic tissues. When the FC value is considered to be 2 for the significance of the expression differences, increased expression of 22 genes (MMP1, MMP12, S100A2, S100A3, CK14, CK16, SLC2A1, ITGA6, CEP55, KLK6, KRT17, LAMC2,

IL1F9, TP63, VSNL1, CXCL1, COL4A5, COL4A6, FSCN1, KRT6B, WDR66 and ACOT7) and decreased expression of two genes (ITM2A and CFD) were observed. Fourteen of 24 genes were known to be involved in the etiology of laryngeal tumors in the literature, but as far as we know, the effect on laryngeal cancer of the remaining 10 genes (VSNL1, CXCL1, COL4A5, COL4A6, FSCN1, KRT6B, WDR66, ACOT7, ITM2A, CFD) was identified for the first time in our study. Differentially expressed genes between LSCC and adjacent normal tissues are shown in Table 2. The hierarchical clustering of the expression of these genes is shown in Figure 1.

Table 1. Clinicopathological characteristics of cases.

Sex, n (%)	
M	12(100)
F	0 (0)
Age	60,8(range 37-86, median value: 61, standard deviation: 14.11)
Tumor localization, n (%)	
supraglottic	3(25%)
glottic	5(41%)
transglottic	4(33%)
subglottic	0(0)
Alcohol and tobacco consumption, n (%)	
Tobacco	7(58%)
Alcohol	3(25%)
Histology	
Squamous cell cancer	12(100%)

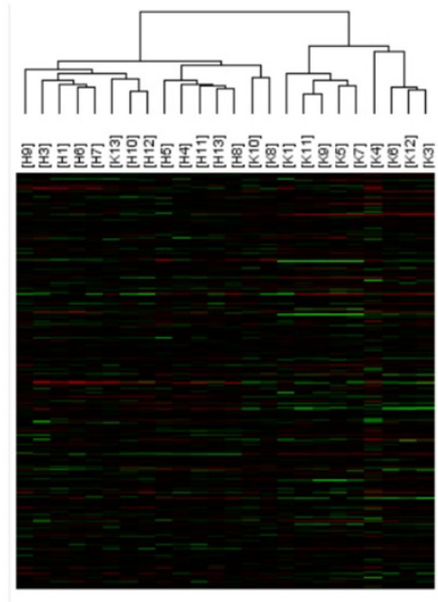

Figure 1. Hierarchical clustering heatmap analysis of the normalized data. The heatmap represents differentially expressed genes between laryngeal squamous cell carcinoma samples and non-neoplastic samples. Gene expression differences are shown in green (upregulation) and red (downregulation) colors. Black areas show no significant change in the indicated genes. 'Euclidean' as a distance unit and 'Wards' as a connection unit were selected.

Table 2. Differentially expressed genes between LSCC and adjacent normal tissues.

Gene symbol	Gene name	Ontology no	Functions
CFD	Hsa complement factor D (adipsin)	GO:0006508	Proteolysis
ITM2A	Hsa integral membrane protein 2A	GO:0016021	Integral Component of Membrane
S100A3	Hsa S100 calcium binding protein A3	GO:0008270	Zinc Ion Binding
MMP12	Hsa matrix metallopeptidase 12	GO:0006508	Proteolysis
SLC2A1	Hsa solute carrier family 2 member 1	GO:0055085	Transmembrane Transport
VSNL1	Hsa visinin-like 1	GO:0005509	Calcium Ion Binding
CXCL1	Hsa chemokine (C-X-C motif) ligand 1	GO:0030036	Actin Cytoskeleton Organization
S100A2	Hsa S100 calcium binding protein A2	GO:0043542	Endothelial Cell Migration
MMP1	Hsa matrix metallopeptidase 1	GO:0030574	Collagen Catabolic Process
CK-14	Hsa similar to Keratin, type I cytoskeletal 14	---	---
CK-16	Hsa similar to Keratin, type I cytoskeletal 16	---	---
KLK6	Hsa kallikrein-related peptidase 6	GO:0006508	Proteolysis
KRT17	Hsa keratin 17	GO:0008544	Epidermis Development
CEP55	Hsa centrosomal protein 55kDa	GO:0007049	Cell Cycle
ITGA6	Hsa integrin, alpha 6	GO:0007044	Cell-Substrate Junction Assembly
ACOT7	Hsa acyl-CoA thioesterase 7	GO:0006629	Lipid Metabolic Process
COL4A5	Hsa collagen, type IV, alpha 5	GO:0005201	Extracellular Matrix Structural Constituent
TP63	Hsa tumor protein p63	GO:0002347	Response to Tumor Cell
COL4A6	Hsa collagen, type IV, alpha 6	GO:0030198	Extracellular Matrix Organization
FSCN1	Hsa fascin homolog 1, actin binding protein	GO:0008283	Cell Proliferation
LAMC2	Hsa laminin, gamma 2.	GO:0007155	Cell Adhesion
WDR66	Hsa WD repeat domain 66	---	---
IL1F9	Hsa interleukin 1 family, member 9	GO:0007267	Cell-Cell Signaling
KRT6B	Hsa keratin 6B	GO:0007398	Ectoderm Development

Hsa: Homo sapiens

Table 3. WikiPathway functional enrichment analysis of some of the differentially expressed genes in LSCC.

Upregulated Pathways	
WikiPathway Term	P-value
Hs_Cell_Cycle_WP179_70629	4,12E-11
Hs_Degradation_of_the_extracellular_matrix_WP2774_8320812	5,44E-11
Hs_Regulation_of_DNA_replication_WP1898_8324929	1,39E-11
Hs_DNA_Replication_WP466_7998116	2,13E-13
Hs_G1_to_S_cell_cycle_control_WP45_80001	1,33E-10
Hs_Cell_Cycle_Checkpoints_WP1775_83240	7,81E-11
Downregulated pathways	
WikiPathway Term	P-value
Hs_Electron_Transport_Chain_WP111_82216	8,94E-11
Hs_Oxidative_phosphorylation_WP623_79961	7,94E-33
Hs_Integrin-mediated_Cell_Adhesion_WP185_80036	1,65E-07
Hs_TCA_cycle_and_respiratory_electron_transport_WP2766_8338525	2,27E-23
Hs_Mitochondrial_translation_WP3310_83252	1,13E-12

Also, to determine the biological functions of differentially expressed genes, WikiPathway functional enrichment analysis was carried out using the Gene Spring gx (Agilent) program. WikiPathways was established to contribute to the information about the pathway and to facilitate the access by the biology community. WikiPathways is a new database including and complementing databases such as KEGG, Reactome and Pathway Commons.

The pathways such as cell cycle, G1 to S cell cycle control, cell cycle checkpoints, regulation of DNA replication, DNA replication, degradation of the extracellular matrix were upregulated; however, the pathways such as electron transport chain, oxidative phosphorylation, integrin mediated cell adhesion, the citric acid cycle and respiratory electron transport, adipogenesis, mitochondrial translation, fatty acid triacylglycerol and ketone body metabolism were downregulated. The WikiPathway analysis of the most affected pathways is shown in Table 3.

Discussion

Cancers occur as a consequence of accumulation of genetic aberrations. Almost every neoplasm has its own unique molecular changes. Characterization of these changes is extremely important in matters of clinical care and the development of personalized treatment strategies.

Laryngeal carcinomas have been identified as aggressive tumors because of their high recurrence and metastasis rate. The first step for regional recurrence and distant metastasis is the deterioration of the extracellular matrix components. Matrix metalloproteinases (MMP) are a family of enzymes that play a key role in this process. Overexpression or dysregulation of these enzymes is known to be related to various types of cancer [6]. Krecicki et al. demonstrated strong immunoreactivity of MMP1 in 36(72%) of 50 laryngeal squamous cell carcinoma cases using immunohistochemical methods [7]. Liu et al. reported a difference in MMP12 expression between tumor and normal tissues, also metastatic and non-metastatic tumors [8]. Kallikrein-related peptidase 6 (KLK6), a member of the serine protease family, is involved in cellular processes such as the degradation of the extracellular matrix in a similar way to MMP. In a study of 162 patients with head and neck tumors, KLK6 overexpression was detected in 42.6% of the cases [9,10]. In addition, it has been shown in many cancers, including LSCC carcinoma, that overexpression of the ITGA6 gene, which is involved in cell-cell and cell-matrix interactions, is also effective in tumor invasion and metastasis [11,12]. Similarly, laminin, which is encoded by the LAMC2 gene, which plays a role in cell differentiation, migration and metastasis, was found to be overexpressed in squamous laryngeal cancer cases by microarray technique [13]. We also detected upregulated expression in the MMP1, MMP12, KLK6, LAMC2 and ITGA6 genes in all of our cases.

S100 proteins are one of the components of the Epidermal Differentiation Complex and are known to be related with skin diseases and human cancers, including squamous cell laryngeal cancer, lung, ovarian, renal, colorectal, skin and gastric carcinomas. In the study by Tyszkiewicz et al., while S100A1 and S100A4 genes were down-regulated, S100A2, S100A3, S100A4 and S100A11 genes were found to be significantly up-regulated in 93 cases of head and neck cancer. In our study, we determined that, S100A2 and S100A3 genes were upregulated in all cases [14].

CK14, CK16, KRT17 and KRT6 genes, encoding members of the keratin family, are responsible for the structural integrity of epithelial cells. Lauriola et al. reported that CK 14 expression difference was detected in 54 of 62 laryngeal carcinoma cases by immunocytochemistry, and this expression was strictly associated with S100A2 [15]. Elazezy has shown that keratin 16 overexpression might be related to more aggressive breast cancer [16]. Khanom R. et al. demonstrated that KRT6 and KRT17 were overexpressed in oral squamous cell carcinoma (OSCC) cell lines [17].

CEP55 (centrosomal protein 55kDa) has been identified as a tumor-associated antigen that functions as a regulator in the PI3K/AKT pathway. Its upregulation has been shown to be associated with poor prognosis in various neoplasms [18]. In a study of laryngeal squamous cell carcinoma cases, expression of CEP55 was found to be increased in early and advanced stages of the tumor [19]. Our results also support the oncogenic role of CEP55 in the development of LSCC. IL1F9 (interleukin 1 family, member 9) activates the NF-kappa B and PI3K pathways. As a result of this pathways' activation, cell growth, differentiation and cell survival increase, while apoptosis is suppressed [20]. PI3K pathway is upregulated in over 90% of head and neck carcinoma cases [21].

In recent years, the Glut-1 protein, encoded by the SLC2A1 gene, has become more and more popular in cancer research areas. Starska et al. observed that SLC2A1 gene expression was upregulated in 83% of 106 laryngeal carcinoma cases and it was associated with poor prognosis [22]. Another study showed that an increase in SLC2A1 gene expression was observed in 30 of 38 head and neck carcinoma patients [23]. In our study, SLC2A1 gene expression was significantly increased, compatible with the literature. TP63, P53 tumor suppressor gene homolog, is highly expressed in various cancers, including laryngeal squamous cell carcinoma [24].

The effect of the genes mentioned above on the etiology of

laryngeal cancer has been previously shown in the literature. Although genes including VSNL1, CXCL1, COL4A5, COL4A6, FSCN1, KRT6B, KRT17, WDR66, ACOT7, ITM2A and CFD were known to be altered in other human cancers, they have not been described before in laryngeal squamous cell carcinoma.

Type IV collagen protein plays an important role in migration and adhesion. Therefore, degradation of type IV collagen is related to cancer progression, invasion and metastasis. In a study with colorectal cancer patients, a loss of expression of COL4A5 and COL4A6 was observed due to promoter hypermethylation of genes [25]. Additionally, in well-differentiated esophageal squamous cell carcinoma specimens, the increased expression of COL4A5 and COL4A6 was found [26]. Similarly, FSCN1 (fascin homolog 1) gene encodes a protein that participates in cell migration, motility, adhesion and cellular interactions. Overexpression of FSCN1 causes metastasis of certain types of cancers, via increasing cell motility [27]. Also, increased expression of FSCN1 was detected in 129 cases of oral and oropharyngeal carcinoma [28].

VSNL1 (Visinin-like protein-1) has been identified as a tumor suppressor in a study of esophageal cancer cases, in contrast to a neuroblastoma study, which described it as an oncogene. In addition, VSNL1 can be used as a marker for Alzheimer's disease and some other neurodegenerative diseases [29,30]

The encoded protein by CXCL1(C-X-C motif chemokine ligand 1) gene takes part in inflammation and acts as a chemoattractant for neutrophils. It is also known that this gene has a tumorigenic, mitogenic and angiogenic effects. Abnormal expression of the protein is associated with the development and progression of melanomas [31]. Also, ACOT7(acyl-CoA thioesterase 7) gene participates in the development of several types of neoplasms including melanomas. ACOT7 upregulation is also associated with poor prognosis in acute myeloid leukemia cases [32,34].

WDR66(WD repeat-containing protein 66) is a large family of proteins involved in various pathways like signal transduction, apoptosis, cell cycle control, transcription regulation and autophagy [35]. Studies have found that WDR66 is overexpressed in gastric carcinoma, thyroid, lung and esophagus carcinomas, and it has been emphasized that these proteins may be tumor markers for these cancers [36,38]

The role of ITM2A in the cellular compartment and in tumorigenesis is currently unknown. However, Nguyen et al. showed that the expression of ITM2A was decreased in cancerous ovarian tissues, and this was interpreted as that this protein might function as a tumor suppressor [39]. CFD has an anti-inflammatory effect through the complement system pathway. Ye H et al. detected decreased CFD gene expression in tongue squamous cell carcinoma cases [40].

In this study, an analysis of expression-altering genes and their pathway analysis were performed. The cell cycle and extracellular matrix degradation pathways were over-represented, while the electron transport chain pathway was under-represented. All affected pathways are shown in Table 3.

Conclusion

We have identified several differentially expressed genes and described their functions in the cell and the affected pathways in laryngeal squamous cell carcinoma. These genes may be used as new biomarkers for diagnosis, prognosis and therapy of LSCC. It is also believed that these genes will shed light on the studies that we wish to do in the future.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

Funding: *Financial support was provided by the Scientific Research Projects of the Necmettin Erbakan University (Project Number: 151518008).*

Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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How to cite this article:
Emine Göktaş, Kayhan Öztürk, Ayşe Gül Zamani, Mahmut Selman Yıldırım. Determination of whole-genome expression differences in larynx cancers. Ann Clin Anal Med 2023;14(Suppl 1):S31-36

This study was approved by the Ethics Committee of Necmettin Erbakan University (Date: 2015-03-13, No: 2015/146)